

## REVERSIBILITY OF CELL SURFACE LABEL REARRANGEMENT

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### ABSTRACT

Cell surface labeling can cause rearrangements of randomly distributed membrane components. Removal of the label bound to the cell surface allows the membrane components to return to their original random distribution, demonstrating that label is necessary to maintain as well as to induce rearrangements. With scanning electron microscopy, the rearrangement of concanavalin A (con A) and ricin binding sites on LA-9 cells has been followed by means of hemocyanin, a visual label. The removal of con A from its binding sites at the cell surface with alpha-methyl mannoside, and the return of these sites to their original distribution are also followed in this manner.

There are labeling differences with con A and ricin. Under some conditions, however, the same rearrangements are seen with both lectins. The disappearance of labeled sites from areas of ruffling activity is a major feature of the rearrangements seen. Both this ruffling activity and the rearrangement of label are sensitive to cytochalasin B, and ruffling activity, perhaps along with other cytochalasin-sensitive structure, may play a role in the rearrangements of labeled sites.

Evidence from a number of laboratories indicates that the application of label to cell surfaces causes a rearrangement of randomly distributed membrane molecules (12, 15, 20, 30, 35, 43, 51, 53). Rearrangement is not a general membrane phenomenon, but involves only the labeled sites; surface molecules which do not interact with the label remain in a random, homogeneous distribution (29). It is not known how the interaction between label and receptor causes rearrangement.

Two components can be recognized during label-induced rearrangement (12, 35, 53, 55): the formation of clusters of label; and the preferential relocation of label to certain areas on the cell and away from others, e.g., capping. It has been proposed that the first component, clustering, results from crosslinking of surface sites by multivalent label molecules, since univalent antibody

fragments appear uniformly distributed on the cell surface (16, 35, 53). Stackpole et al. (50), however, have demonstrated that clustering can occur even with strictly univalent reagents. They suggest that interaction of label with its binding sites may cause an alteration which thermodynamically favors aggregation. The second component of rearrangement must involve more than crosslinking or aggregation of labeled molecules in the plane of the membrane; it requires energy (12, 15, 20, 35, 53, 57) and must therefore be directed by some activity of the cell.

In this communication we follow the rearrangements at the cell surface obtained with two different lectins and show that the label-receptor interaction is necessary for the maintenance as well as the induction of rearrangements. Mechanisms consistent with our findings whereby label-receptor

interaction could give rise to rearrangements are discussed.

## MATERIALS AND METHODS

### Reagents

Concanavalin A (con A) is obtained from Sigma Chemical Co., St. Louis, Mo., and purified by the method of Agrawal and Goldstein (4). It is extensively dialyzed against distilled water, lyophilized, and stored at  $-20^{\circ}\text{C}$ . Alpha-methyl mannoside and galactose are also obtained from Sigma.

Hemocyanin is obtained from the whelk *Busycon canaliculatum* (Woods Hole Marine Biological Laboratory, Woods Hole, Mass.) by breaking the shell with a hammer in the region of the heart and allowing the hemocyanin to drip out into a beaker. After a low-speed centrifugation to remove shell fragments and other debris, the hemocyanin is concentrated by a high-speed centrifugation (30 min  $\times$  57,000 g in a Ty 40, 50, or 65 rotor). This treatment does not pellet most of the hemocyanin; excessive centrifugation forms a pellet which is hard to dissolve. The most concentrated portion may be collected visually, as oxygenated hemocyanin is blue. It is then purified by passage through a Sepharose 2B column (Pharmacia Fine Chemicals, Piscataway, N.J.). Concentration is estimated by the procedure of Lowry et al. (36).

Ricin (RCA<sub>1</sub>; 39) is a gift of Dr. Charles Birdwell, California Institute of Technology. It was coupled to hemocyanin with glutaraldehyde by the method of Avrameas (5). The reaction mixture contained 3 mg/ml ricin and 20 mg/ml hemocyanin in PBS, which was made  $10^{-2}$  M in galactose to protect the active site of ricin during the coupling reaction. 0.5% glutaraldehyde was then added slowly, while stirring, to a final concentration of 0.05%. After 2 h, glycine was added to a final concentration of 0.05 M for 0.5 h, and the mixture was dialyzed against PBS. The conjugate was purified by affinity chromatography: ricin and the conjugate interact with a Sepharose 2B column whereas unconjugated hemocyanin passes through. Ricin and the conjugate are then eluted separately with 0.1 M galactose, a hapten inhibitor of ricin.

It is not necessary to couple con A to hemocyanin since they react with each other. Con A bound to the cell surface has remaining active sites which can interact with hemocyanin (48). Cytochalasin B (Calbiochem, San Diego, Calif.) is dissolved in dimethyl sulfoxide to give a 1 mg/ml stock solution. Phosphate-buffered saline (PBS) (18) is used at a pH of 7.4.

### Labeling Procedures

LA-9 cells (34) are grown on glass cover slips in Eagle's minimum essential medium plus 10% calf serum (MEM) (Grand Island Biological Co., Grand Island,

N. Y.). All incubations with labeling solutions are performed at  $37^{\circ}\text{C}$  by placing the cover slips on a baffled metal table through which water from a constant temperature bath is circulated. Cells are rinsed briefly in PBS, incubated for 1–10 min (10 min is used unless otherwise specified) in 100  $\mu\text{g}$  of con A per ml of PBS, rinsed in PBS, and labeled for 10 min with 1 mg/ml hemocyanin in PBS. The hemocyanin incubation can take place either before or after fixation, but blocking (see below) is required in the latter case. This procedure results in the labeling of con A binding sites with hemocyanin (48), a visual marker which can be recognized in the scanning electron microscope (8, 38, 41, 42, 58).

The labeling procedure for ricin is similar, except that since the ricin and hemocyanin are coupled together, labeling takes place in one instead of two incubations. Cells are rinsed in PBS, incubated in 1 mg/ml of the ricin-hemocyanin conjugate, and rinsed before fixation.

After labeling the cells are fixed at  $37^{\circ}\text{C}$  for 10 min in 1% glutaraldehyde in PBS. If cells are to be relabeled or labeled for the first time after fixation, they are incubated overnight at  $0-4^{\circ}\text{C}$  in 0.1 M ammonium chloride in PBS to block any remaining aldehyde groups which might otherwise cause nonspecific labeling. They are then labeled and fixed as described for unfixed cells.

Controls for these labeling procedures include the appropriate hapten inhibitor of lectin binding, alpha-methyl mannoside for con A and galactose for ricin, to demonstrate the specificity of labeling. Cells were incubated for 10 min at  $37^{\circ}\text{C}$  in 0.01–0.1 M hapten inhibitor, either with or after the lectin incubation.

In the experiments testing the reversibility of rearrangement, cells are treated for 10 min with 100  $\mu\text{g}$ /ml of con A, and then washed for 1–10 min in PBS, alone or containing 0.01–0.1 M galactose or alpha-methyl mannoside. The cells are then fixed and relabeled as described above to assess the degree to which rearrangement has been reversed, or fixation is followed by hemocyanin only, to demonstrate how much con A has been removed.

For cytochalasin experiments, cells are incubated with 1–10  $\mu\text{g}$  of cytochalasin B per milliliter of MEM for at least 1 h. All subsequent incubations in labeling solutions before fixation include a like amount of cytochalasin B. Control experiments contain a like amount of dimethyl sulfoxide, but cytochalasin B is omitted.

### Preparation for the Scanning Electron Microscope

Cells are postfixed for 30 min at  $0-4^{\circ}\text{C}$  in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4. Dehydration is accomplished by 2-min long, 10% steps from 40% to 100% ethanol, then three changes of 100% ethanol of 15 min each. The cells are dried from Freon 13 by the "critical point" method (11), shadowed at  $10^{-4}$  torr with 5 cm of gold wire (8 mil) at a distance of 8–10 cm from the sample on a rotary stage, and stored in a desiccator. They are examined in a scanning electron microscope.

## RESULTS

### *Distribution of Con A Binding Sites*

When LA-9 cells are treated sequentially with con A and hemocyanin, the hemocyanin label can be observed on their surfaces by scanning electron microscopy (8, 38, 41, 42, 58). This labeling is specific for con A binding sites, as it can be removed or prevented with alpha-methyl mannoside, a hapten inhibitor of con A binding (26).

Cells are fixed before labeling to avoid alterations in the distribution of surface sites caused by the labeling procedure (7, 12, 42, 43). Under these conditions the hemocyanin is distributed evenly over the surface of all cells observed (Fig. 1 *a*), indicating that unlabeled con A binding sites have a random arrangement. These cells demonstrate the normal morphology of unlabeled LA-9 cells, including ruffling activity (1) at one or more locations on the cell (Fig. 1 *a*).

### *Effect of Labeling on Distribution of Con A Binding Sites*

Labeling cells which have not been fixed beforehand causes alterations in the morphology of the cell and in the distribution of con A binding sites (Fig. 1 *b*). As shown by time-lapse cinematography as well as scanning electron microscopy, 1–2 min of incubation in con A before fixation results in a sizable reduction in the number of ruffles extended up at an angle to the substrate; instead, most cell extensions are parallel to the substrate, and appear stuck to it. (These processes resemble the first stage in ruffle formation, which is a horizontal extension of membrane. What would ordinarily occur next is the movement of this flat extension to a more vertical position [28].) Since it can be observed in these experiments that con A reacts with sites on the substrate as well as on the cell, it is possible that con A is crosslinking the ruffle to the substrate, preventing the usual upward movement. With exposure to con A for even as brief a period as 1 min, the first signs of a rearrangement of con A binding sites can be seen. They clear preferentially from those processes which appear attached to the substrate. Ruffles extending upwards from the cell body are mostly labeled.

The ruffling activity has ceased on most of the cells by 4 min of incubation in con A. The periphery of the flattened processes has become irregular, as if they had retracted somewhat, leaving regions protruding. The areas of mem-

brane cleared of label are better delineated, and microvilli as well as peripheral processes are now unlabeled. Some of the label has come together in clusters away from the cell periphery.

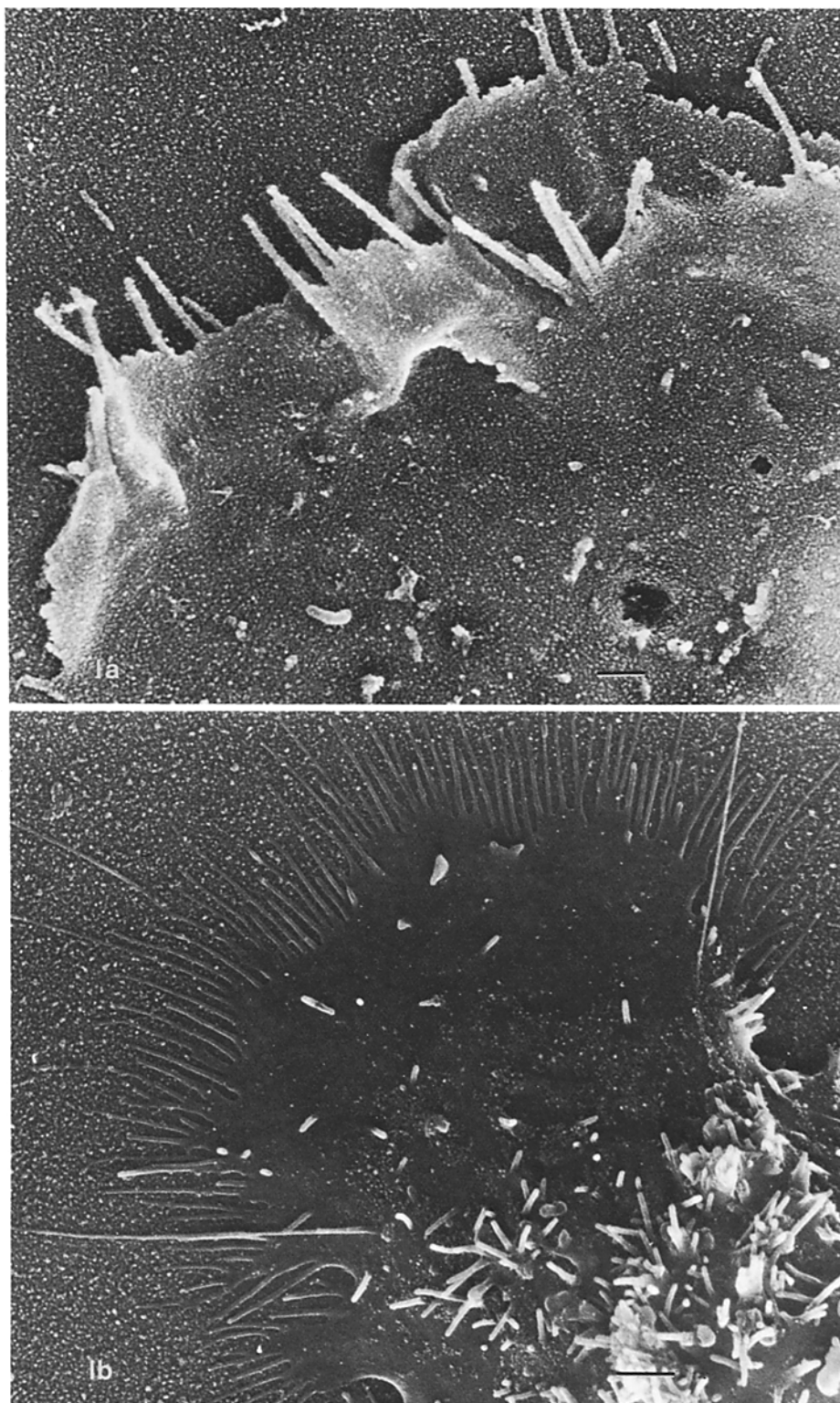
Further incubation in con A (up to 1 h) does not dramatically alter this picture, but heightens the pattern already described. After 10 min of incubation in con A (Fig. 1 *b*), the clusters of con A binding sites have become more closely packed. At times the label is now also absent in the central region of the cell and is concentrated in a perinuclear ring. No ruffling activity can be recognized as such. The appearance of cells and distribution of label is the same whether cells are fixed immediately after exposure to con A for 10 min or incubated for an additional 10 min with hemocyanin or PBS.

Cells in control experiments in which PBS alone is substituted for the con A closely resemble the prefixed cells described in the previous section. Flattened processes and rearrangement of con A binding sites (upon labeling after fixation) are not seen, although the incubation in PBS before fixation does result in some reduction in the size of the ruffles.

### *Effect of Label Removal on Distribution of Con A Binding Sites*

Alpha-methyl mannoside is a hapten inhibitor of con A (26). It can be used to remove con A bound to the cell surface. The removal can be followed by the disappearance of hemocyanin labeling. Cells are labeled for 10 min with con A, resulting in the rearrangements (Fig. 1 *b*) described in the previous section. They are then incubated for 1–10 min in 0.01 M alpha-methyl mannoside, fixed, excess fixative neutralized (blocking, see Materials and Methods), and exposed to hemocyanin. Very little of the con A appears to have been removed in 1 min, whereas a significant amount has been removed in 2 min. After 4 min of treatment with alpha-methyl mannoside, most of the con A is removed, and after 10 min, almost no hemocyanin binds to the cell surface. Alpha-methyl mannoside also removes con A from the substrate, although not so efficiently.

Removal of the con A allows a concomitant return of the binding sites to the original, random distribution described on cells fixed before exposure to label. This process is observed as follows: cells are treated for 10 min with con A, then with alpha-methyl mannoside for the same time periods



**FIGURE 1** Distribution of con A binding sites on LA-9 cells labeled with (1 *a*) or without (1 *b*) prefixation in glutaraldehyde. In both cases, cells were treated for 10 min at 37°C with con A, followed by hemocyanin. The resulting patterns of label seen are quite different. In 1 *a*, where fixation prevents label-induced rearrangement of binding sites, the label is distributed homogeneously. Active ruffling can be seen at the periphery of this cell. In 1 *b*, which has not been prefixed, the label demonstrates a number of rearrangements. The label is absent from the microvilli and the periphery of the cell, which ceases to ruffle, becomes flattened, and appears retracted. Label seen on the background is probably largely due to adsorption of serum to the glass cover slip on which the cells are grown. 1 *a*,  $\times 7,200$ ; 1 *b*,  $\times 8,000$ . Bar = 1  $\mu\text{m}$  in all figures.

as above, and fixed. The distribution of binding sites after removal of con A is assessed by relabeling the preparation with con A after fixation and blocking, when no further rearrangements can occur. Hemocyanin is then used to label con A at the cell surface, revealing both that applied after and that remaining from before fixation. Little evidence of a return to a homogeneous distribution is seen after 1 min of incubation in alpha-methyl mannoside, an exposure which does not remove much of the con A from the cell surface. After 2 min of exposure to alpha-methyl mannoside, however, when a fair amount of the con A has been removed, there is some return of labeling in the cleared periphery. There is also a variable amount of labeling on microvilli. By 4–10 min in alpha-methyl mannoside, when most of the original con A has been removed, the labeling appears homogeneous, having completely returned to peripheral processes and microvilli (Fig. 2 *a*). The peripheral processes appear to be less retracted with increasing alpha-methyl mannoside incubation, implying that removal of con A allows a recovery of ruffling activity as well as a return of con A binding sites to a native arrangement. Any ruffling seen after 10 min of incubation with alpha-methyl mannoside is of very small magnitude, however, the recovery process being slow compared to the rate at which sites become randomized. Since randomization goes on concurrently with con A removal, it cannot be timed precisely but takes less than 4 min and may take even less than 1 min.

Controls demonstrate that the removal of con A is necessary for the changes described. If alpha-methyl mannoside is omitted from the 10-min wash, or replaced with galactose (conditions under which con A is not removed from the cell surface), a random distribution is not seen (Fig. 2 *b*). A much longer PBS wash (minimum of 1 h; reference 8) is required for significant appearance of new, unlabeled sites in cleared areas (detected by labeling after fixation).

#### *Effect of Cytochalasin B on Distribution of Con A Binding Sites*

Addition of 1  $\mu\text{g/ml}$  of cytochalasin B to the culture medium does not affect the normal distribution of con A binding sites (Fig. 3 *a*). This concentration also does not have gross morphological effects, although ruffling activity is reduced (as it is during incubation in PBS; see above), even

before incubation with con A, which further reduces ruffling.

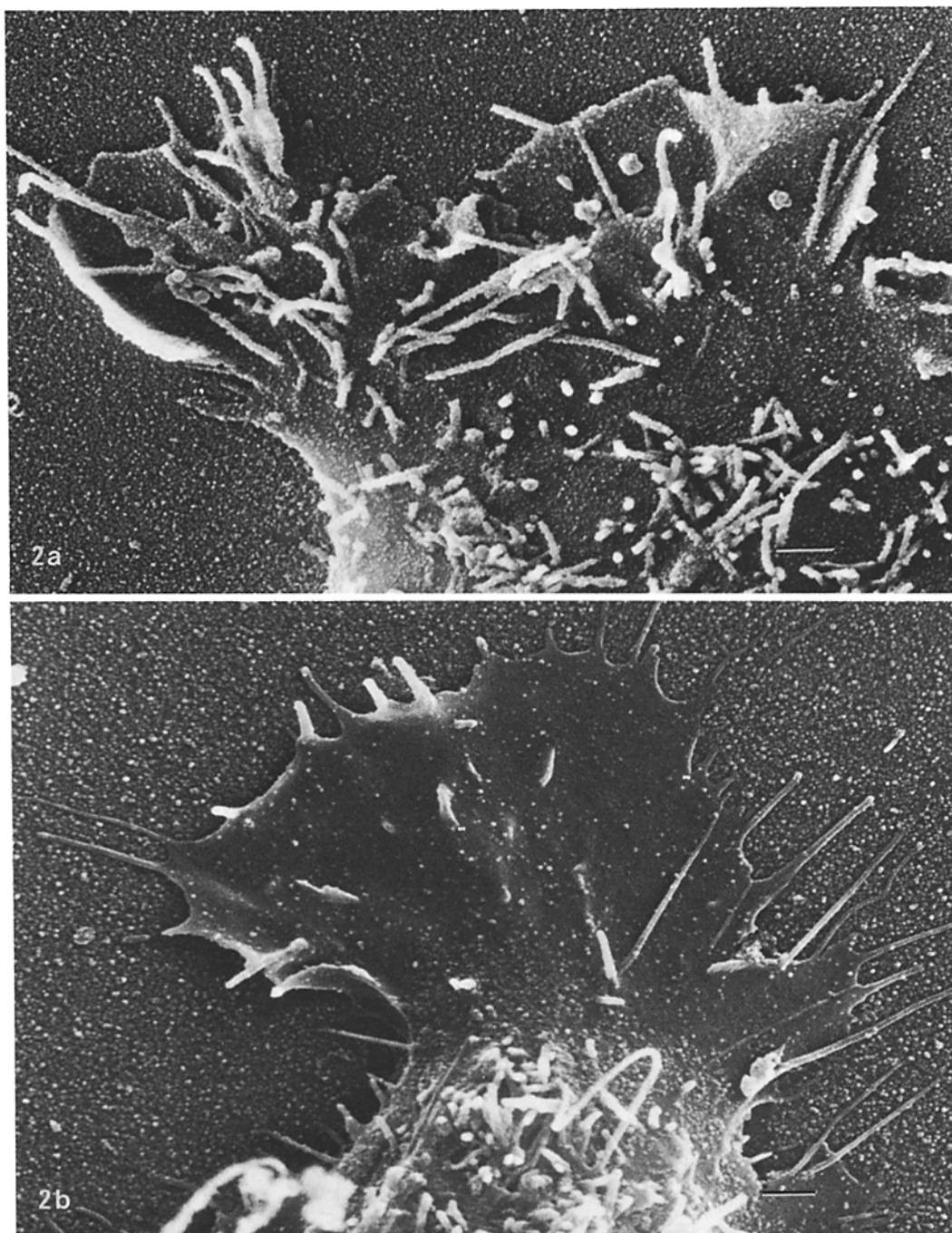
Treatment with 10  $\mu\text{g/ml}$  of cytochalasin B, on the other hand, prevents clearing of label from the cell's edges; the label is found in large patches all over the cell (Fig. 3 *b*). The microvilli remain unlabeled for the most part. Typically the cell bodies round up, with cytoplasmic processes remaining attached to the substrate (6, 23, 25, 41, 49). All ruffling activity is abolished, and other distortions of LA-9 morphology are seen, such as clustering of microvilli and blebbing (6), sometimes in the areas of former ruffling activity. Label is often associated with these clusters of microvilli.

Controls without cytochalasin B but with corresponding concentrations of dimethyl sulfoxide (used to dissolve cytochalasin B) show no observable effects on cell morphology or labeling pattern.

#### *Distribution of Ricin Binding Sites*

Incubation of fixed LA-9 cells with a ricin-hemocyanin conjugate also results in a homogeneous labeling pattern. This labeling is specific as it can be removed with galactose, the hapten inhibitor of ricin binding (17). Unlike con A, however, the ricin-hemocyanin conjugate does not cause morphological changes upon incubation with unfixed cells. LA-9 cells still exhibit ruffling activity after 10–30 min of incubation with the ricin label. Furthermore, the distribution of ricin binding sites still appears homogeneous on all cells observed under these conditions (Fig. 4 *a*). If, however, labeling is followed by a 10-min PBS wash, rearrangements similar to those described for con A are seen (Fig. 4 *b, c*). Ruffles and microvilli are essentially unlabeled, and there is a patchiness of labeling in other regions of the cell.

All of the areas which are unlabeled after washing can be immediately relabeled, either before or after fixation (Fig. 4 *d*). This implies that the same sorts of rearrangements of labeled sites are going on in the presence of con A and ricin-hemocyanin, but that they are masked in the latter case by unlabeled sites appearing in areas from which labeled sites are becoming cleared. In the presence of ricin, these unlabeled sites themselves become labeled, yielding a uniformly labeled appearance. The source of these unlabeled sites is currently being investigated with the use of radioactive label. These studies suggest that a major class of ricin binding sites are saturated under the conditions we have used, but they do



**FIGURE 2** Effect of label removal on the distribution of con A binding sites. Cells were treated for 10 min at 37°C with con A, followed by a 10-min wash at 37°C in either 0.01 M alpha-methyl mannoside in PBS (2 *a*), or PBS only (2 *b*). Cells were then fixed in 1% glutaraldehyde, blocked overnight in 0.1 M ammonium chloride, and then relabeled with con A and hemocyanin, under the same conditions as in Fig. 1. Alpha-methyl mannoside was included in the wash (2 *a*) to remove the con A which had already bound to the cell surface and caused a rearrangement of binding sites. The relabeling revealed that the binding sites were then present homogeneously, as in Fig. 1 *a*. In the control (2 *b*) which did not include alpha-methyl mannoside in the wash, the periphery remained essentially free of binding sites, as in Fig. 1 *b*. Con A has reduced the ruffling activity of both of these cells. There may be some recovery in 2 *a* upon removal of con A. 2 *a*,  $\times 8,000$ ; 2 *b*,  $\times 7,500$ .

not rule out the possibility of a population of lower affinity binding sites (54).

## DISCUSSION

This report confirms the findings of others that label can cause a rearrangement of surface sites from a homogeneous to a heterogeneous distribution. Con A and ricin binding sites are initially homogeneously distributed on the surface of LA-9 cells, which is demonstrated by fixing the cells before labeling to prevent rearrangements (7, 12, 42, 43). The con A binding sites disappear quickly from peripheral processes and microvilli when unfixed cells are exposed to con A. The same rearrangements are caused by ricin, but are masked under some labeling conditions. Apparently, unlabeled sites continue to appear in areas which are clearing of label and then become labeled as long as ricin is present. It is necessary to pulse, i.e. to have a period without ricin before fixation, to demonstrate the rearrangements of the labeled portion of binding sites.

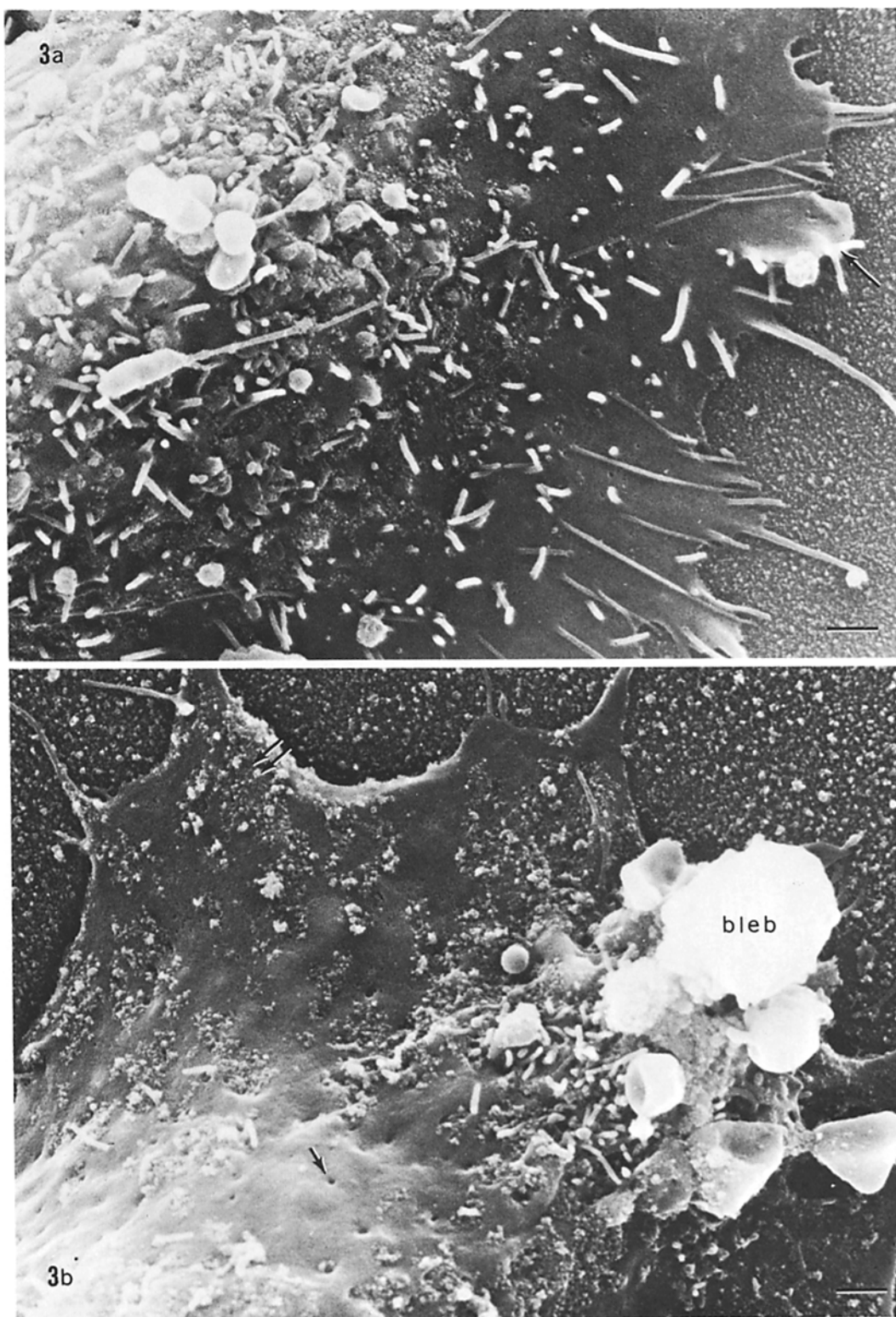
We demonstrate that the rearrangement of con A binding sites is not only caused but also maintained by the interaction of the label with surface sites. Under conditions where con A is allowed to induce a rearrangement, but is then removed by alpha-methyl mannoside, the sites return to their original homogeneous arrangement as the con A is removed. Because control experiments in which alpha-methyl mannoside is omitted or replaced by galactose do not show a return to a homogeneous arrangement, we conclude that it is the removal of con A bound to the cell surface which causes the reversal of the heterogeneous pattern caused by con A. These control experiments demonstrate that the homogeneous pattern seen upon con A removal cannot be an artifact of labeling after fixation. The controls also demonstrate that the homogeneous pattern is not the result of insertion of new con A binding sites, unless removal of con A permits insertion that does not otherwise take place. A minimum of 1 h wash in PBS is required for noticeable label to reappear in cleared areas (7), whereas randomization upon removal of con A takes minutes or less. It is very difficult to imagine that new binding sites could reappear rapidly enough to account for this randomization. Thus, the data obtained suggest that removal of con A allows a return of the pre-existing, previously clumped sites to the unlabeled areas.

This is consistent with the proposed "fluid mosaic" membrane model (47), in which membrane components are freely diffusible in the plane of the membrane. Measured diffusion rates of proteins in membranes (19) are quite rapid and adequate to account for the return of sites to a homogeneous distribution as con A is being removed during the alpha-methyl mannoside wash. A reservation which should be kept in mind, however, is that it has not been proven that the con A binding sites seen with cell surface labeling are integral to the membrane. Interference with rearrangement by cytochalasin B might be taken to suggest that the con A binding sites studied here are part of a membrane molecule, possibly one that might span the whole membrane.

Our study extends the findings of others at the light microscope level. Studies of capping of con A-fluorescein on lymphocytes show that a preformed "cap" of label disperses in the presence of metabolic inhibitors (45) and cytochalasin B (14), agents which have previously been shown to interfere with capping (12, 20, 35, 53). Ukena et al. (55), on the other hand, find that cytochalasin B does not reverse the rearrangement of con A and hemocyanin on SV40-transformed 3T3 cells. This may be due to a greater degree of crosslinking between label molecules. Crosslinking can occur in this system not only by con A bridging surface molecules, but also by hemocyanin bridging con A molecules.

In the instances described above, the cellular activity responsible for capping is turned off or interfered with. In our experiment, label is simply removed from the binding sites. This experiment eliminates the possibility that label binding acts as a trigger for a process which does not require label once it is set in motion. At least two possibilities remain for the mechanism of label-induced and maintained rearrangement of surface components. The first is that binding of label and perhaps the subsequent aggregation or crosslinking of surface sites "turns on" a cellular activity which causes rearrangement, and removal of label turns it off. Maintenance of the rearrangement by this mechanism would depend not on the binding or crosslinking by the label per se, but on the continued activity of the stimulated system, which metabolic inhibitors (45) and cytochalasin B (14) could turn off. For example, binding and aggregation of label could stimulate endocytosis and membrane turnover, although de Petris and Raff (15) have shown that these activities in themselves are not adequate







to explain the observed rearrangement. It has been proposed that an intracellular network might be responsible for rearranging surface molecules (15, 35, 50). Binding and aggregation of label could cause and maintain the interaction of the binding sites with this network. This network could then either transfer the message to other parts of the cell or itself direct the rearrangement. de Petris (14) has suggested that such a network could be provided by microfilaments because of the effects of cytochalasin B on label-induced rearrangement. He and others (44, 56) have found a cooperative inhibitory effect of cytochalasin B and colchicine on capping, indicating that a microtubule system may be involved as well. Networks of microfilaments (49) and microtubules (40) are present below the cell surface, but there is no absolutely conclusive evidence for the direct involvement of such networks in the arrangement of surface molecules. The drugs cytochalasin B (9, 10, 21, 27, 31, 33, 35, 46, 52) and colchicine (13, 24, 32, 37) used in some of the experiments above may exert effects independent of these networks.

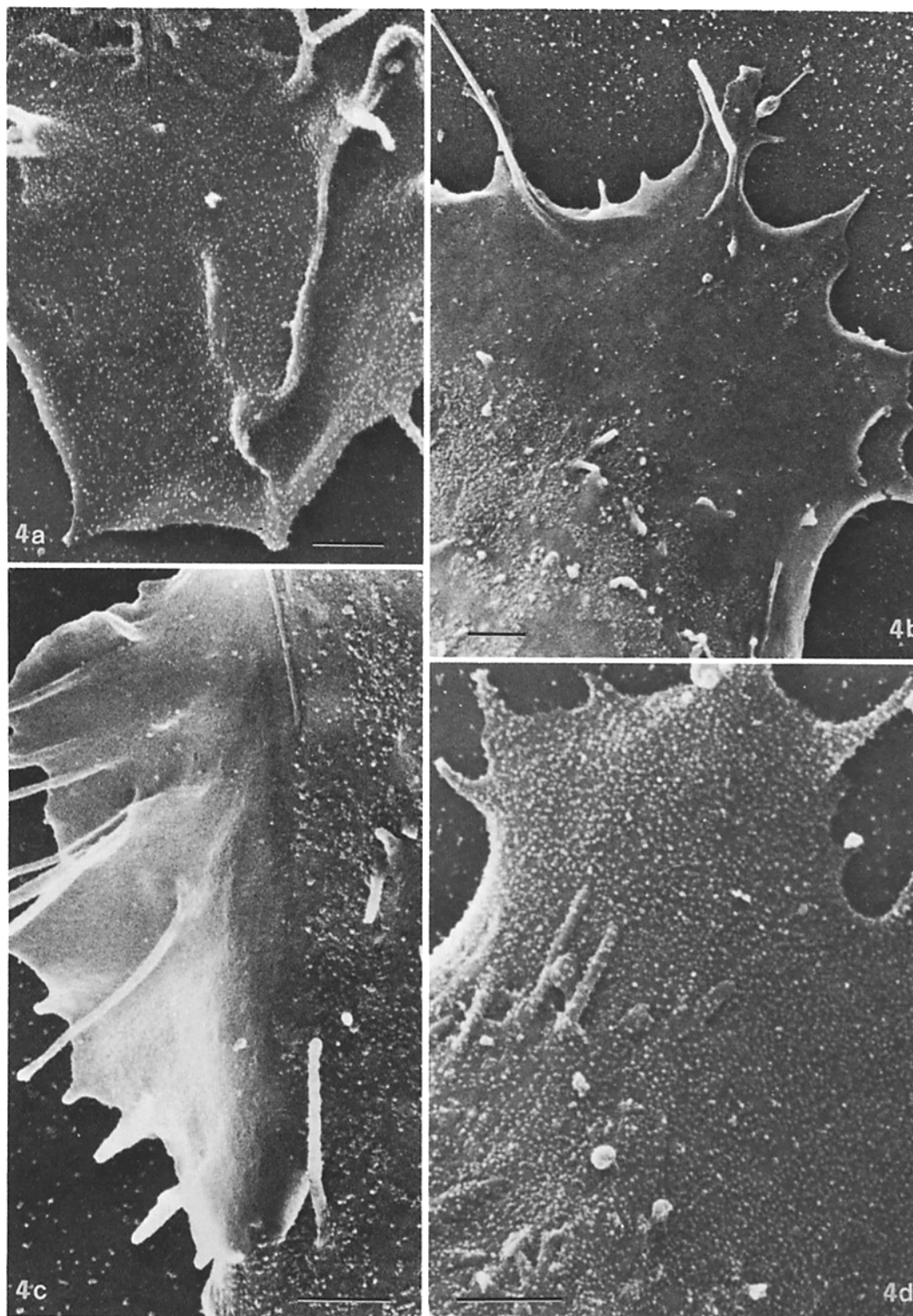
An alternative mechanism is that the activity responsible for rearrangement is not turned on by label, but is going on all the time. Ruffling (1) can be imagined to be part of this mechanism because clearing of label occurs in regions of ruffling activity. This is best illustrated with a pulse of ricin-hemocyanin, which does not affect ruffling activity. With con A the situation is more complicated to interpret, since con A interferes with ruffling activity. The ruffles which can still be seen after brief (1- to 2-min) con A incubations have not become cleared of label in the presence of con A. These ruffles may have formed before the start of the con A incubation, suggesting that clearing may be a function of ruffle formation, rather than of the structure itself. The flattened processes we have described have cleared, and may represent the fate of ruffles forming in the presence of con A.

The distribution of labeled sites could be affected by ruffling activity in several different ways. Unlabeled sites might be able to diffuse freely in the plane of the membrane and flow into forming ruffles along with other membrane components, whereas diffusion of labeled sites would be hindered by the label attached to them and the interaction of labeled sites in the plane of the membrane. Increased crosslinking would then enhance and stabilize the rearrangement, and removal of the label would free the binding sites to return to a random distribution. Alternatively, the anchoring of membrane components to a submembranous network as a result of label binding could be responsible for preventing labeled sites from flowing into forming ruffles; it could simply restrict the mobility of labeled binding sites attached to it, rather than actively directing rearrangement.

Abercrombie et al. (2, 3) suggested that ruffles clear of label because they are formed from new, unlabeled membrane. This is consistent with the ricin-hemocyanin data, but does not explain rearrangements with con A, since areas cleared of con A binding sites cannot be relabeled for at least 1 h. The inhibitory effect of con A on the ruffling process may contribute to the lack of immediate relabeling. Of course, not all membrane components need to be replaced at the same rate.

Our studies with cytochalasin B do not permit us to distinguish between the various mechanisms for label-induced rearrangement proposed above, but have allowed us to make the following observations: both ruffling activity and exclusion of label from the front end of the cell are completely inhibited at 10  $\mu\text{g/ml}$  but not at 1  $\mu\text{g/ml}$ . Clusters of blebs and microvilli form at 10  $\mu\text{g/ml}$  of cytochalasin B and label is often associated with these areas, suggesting that a microfilament network has contracted, carrying along labeled sites and microvilli and forming blebs out of membrane in between.

**FIGURE 3** Effect of cytochalasin B on the distribution of con A binding sites. These cells have been treated with different concentrations of cytochalasin B. Fig. 3 *a* has been incubated in 1  $\mu\text{g}$  of cytochalasin B per ml of MEM, then in con A and hemocyanin solutions in PBS also containing 1  $\mu\text{g/ml}$  of cytochalasin B. The con A binding sites show the normal rearrangement, such as that seen in Fig. 1 *b*. The arrow indicates an area where there may be some ruffling activity. Fig. 3 *b* has been incubated in 10  $\mu\text{g/ml}$  instead of 1  $\mu\text{g/ml}$  of cytochalasin B. Both the morphology and the pattern of label have changed drastically. No ruffling activity is seen, and microvilli can be seen to be clustered in a region which also includes blebs and much label. The label can be present all the way to the edge of the cell (double arrows), but is not homogeneous. Instead, it is found in large clusters all over the cell. Pits (arrow) are presumed to be openings to pinocytotic vesicles, and are also seen on cells which have not been exposed to cytochalasin B.  $\times 8,000$ .



**FIGURE 4** Distribution of ricin binding sites under different labeling conditions. The cell in Fig. 4 *a* has been treated with ricin-hemocyanin conjugate for 20 min at 37°C, and rinsed only briefly (ca. 5 s) before fixation. This picture demonstrates that the front end of the cell is labeled homogeneously under these conditions. In (*b*) and (*c*) the 20-min period of labeling with ricin-hemocyanin is followed by a 10- (*c*) or 15- (*b*) min wash in PBS before fixation. The distribution seen after the wash is heterogeneous, resembling that demonstrated for con A in Fig. 1 *b*. Unlike con A, however, ricin does not affect ruffling activity, as can be especially well appreciated in Fig. 4 *c*. If the treatment described for (*b*) and (*c*) is followed by relabeling after fixation, the appearance illustrated in (*d*) is obtained. Those areas which are clear of label in (*b*) and (*c*) become relabeled with this treatment. (*a*)  $\times 10,500$ ; (*b*)  $\times 8,400$ ; (*c*)  $\times 13,700$ ; (*d*)  $\times 15,300$ .

Clearing of label from microvilli may operate by the same mechanism as clearing from ruffles. These structures, like ruffles, have microfilaments associated with them, and there is some evidence that they, too, are dynamic structures (22).

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